GENETIC CONTROL OF ORGAN ABSCISSION

Related Applications

[0001] This Application is a continuation of International Application PCTUS02/01938, filed January 22, 2002, which claims the benefit of priority under 35 U.S.C. 119(e) to U.S. Provisional Application Serial No. 60/264,974, filed November 29, 2001, the disclosures of which are incorporated by reference herein in their entireties.

Government Interest in the Invention

[0002] Certain aspects of the invention disclosed herein were made with United States Government support under National Science Foundation Grant No. MCB-004-9003 and Department of Energy Grant No. DE-FG03-00ER-15113. The United States Government may have certain rights in the invention.

Field of the Invention

[0003] The invention relates to genetic control of organ abscission in plants. In particular, the invention relates to regulation of floral abscission in plants by the *NEVERSHED* gene, and homologs, variants, and fragments thereof. The invention further relates to the use of *NEVERSHED*, homologs, variants, and fragments thereof, to manipulate floral abscission in a variety of plant species.

Background of the Invention

[0004] Specialized cell types allow plants to shed entire organ systems, such as leaves, flowers, and fruits. The ability to shed organs that have fulfilled their purpose enables plants to make efficient use of nutrients and energy sources. Abscission can also act as a plant defense mechanism: plants can protect themselves from disease by shedding infected organs. Organ shedding sometimes serves a propagative function, as in the seed dispersal promoted by fruit abscission.

[0005] Abscission zones are thought to differentiate as organs form, and can consist of a few to several cell layers of small, densely cytoplasmic cells. Prior to abscission, these cells have been shown to enlarge, and to secrete cell wall hydrolyzing enzymes such as

cellulases and polygalacturonases. Secretion of polygalacturonase causes breakdown of the pectin-rich middle lamella between neighboring cells, thus allowing for cell separation to occur between abscission zone cells. After the organ has been shed, abscission zone cells left behind enlarge and form protective scar tissue (Bleecker and Patterson, 1997, *Plant Cell* 9: 1169-1179, which is incorporated by reference herein in its entirety).

[0006] Studies using abscission zone explants have demonstrated that ethylene promotes abscission (reviewed in Sexton and Roberts, 1982, *Ann Rev Plant Physiol* 33: 133-162; Osborne, 1989, *Crit Rev Plant Sci* 8:103-129, both of which are incorporated by reference herein in their entireties), and ethylene has been linked to abscission zone cell enlargement and zone-specific expression of hydrolytic enzymes (Jensen and Valdovinos, 1968, *Planta* 83: 303-313; Valdovinos and Jensen, 1968, *Planta* 83: 295-302; Wright and Osborne, 1974, *Planta* 120: 163; Koehler *et al.*, 1996, *Plant Mol Biol* 31: 595-606; van Doorn and Stead, 1997, *J Exp Bot* 48: 821-837, all of which are incorporated by reference herein in their entireties). A genetically defined role for ethylene response in the temporal regulation of abscission has been demonstrated by the discovery of ethylene-insensitive mutants such as *etr1* and *ein2* (Bleecker *et al.*, 1988, *Science* 241: 1086-1089; Guzman and Ecker, 1990, *Plant Cell* 2: 513-523, both of which are incorporated by reference herein in their entireties). Studies of these *Arabidopsis* mutants have shown that ethylene insensitivity causes a delay in floral abscission, indicating that ethylene response mediates the timing of programmed cell separation (Bleecker and Patterson, 1997, *Plant Cell* 9: 1169-1179, which is incorporated by reference herein in its entirety). However, abscission does eventually occur in ethylene-insensitive mutants, albeit delayed, indicating that additional pathways must also regulate this process.

[0007] Although numerous studies have addressed hormonal regulation of the abscission process and physiological aspects of abscission zone cell separation, very few studies have focused on regulation of abscission zone development, with the result that the genes whose function is necessary for abscission to occur have not been identified in any plant system. Studies of genetic control of abscission zone development is being carried out using two tomato mutants known as *jointless* and *jointless2* in which formation of pedicel abscission zones in tomato flowers is prevented; molecular characterization of the *jointless* locus is currently in progress (Butler, 1936, *J Hered* 27: 25-26; Wing *et al.*, 1994, *Mol Gen*

Genet 242: 681-688; Zhang *et al.*, 1994, *Mol Gen Genet* 244: 613-621; Szymkowiak and Irish, 1999, *Plant Cell* 11: 159-176, all of which are incorporated by reference herein in their entireties).

[0008] Screens for abscission (*abs*) mutants in the model plant *Arabidopsis* have been carried out and several *abs* mutants have been isolated in which floral abscission is delayed. The lack of mutants for which organ abscission is specifically and completely blocked has limited the progress of studies of abscission zone development in *Arabidopsis*.

[0009] The small GTP-binding protein ARF plays an established role in the control of vesicular traffic and in the regulation of phospholipase D (PLD) activity. GTPase activating proteins (GAPs) are associated with all families of small GTP binding proteins, acting as signal terminators and possibly also in some cases as effectors downstream of the GTP binding protein. The fact that ARF has undetectable intrinsic GTPase activity suggests that the ARF GAP is an essential terminator of ARF-regulated processes.

[0010] ARF has important roles in the control of vesicular traffic and in the regulation of phospholipase D activity. Replacement of bound GDP with GTP produces active ARF-GTP, which can associate with membranes. Both forms are important in vesicular transport, which requires that the ARF molecule cycle between active and inactive states. Like the many other GTP-binding proteins or GTPases that are molecular switches for the selection, amplification, timing, and delivery of signals from diverse sources, ARF functions via differences in conformation that depend on whether GTP or GDP is bound. Vectorial signaling results from the necessary sequence of GTP binding, hydrolysis of bound GTP, and release of the GDP product (Moss & Vaughn, 1998 *Jnl Biol Chem* 273: 21431-21434, which is incorporated by reference herein in its entirety).

[0011] ARF proteins in their GTP-bound form are required for coatomer binding to Golgi stacks and for the binding of clathrin adaptor particles to the trans-Golgi network. GTP hydrolysis is required for the dissociation of these proteins from Golgi-derived membranes and vesicles, a process in which an ARF GAP is most likely involved, indicating that ARF GAPs are involved in vesicle coat disassembly as an uncoating factor.

[0012] Vesicular transport has been extensively studied in the Golgi and ER-to-Golgi pathways (Cosson & Letourneur, 1997 *Curr. Opin. Cell Biol.* 9: 484-487, which is incorporated by reference herein in its entirety). The mechanisms, including the molecules

and their functions, are likely very similar in other pathways. Formation of a transport vesicle begins when activated ARF with GTP bound to it associates with the cytoplasmic surface of a donor membrane. Activated ARF interacts with a coat protein, one of seven in the coatomer complex. Recruitment of multiple ARF molecules followed by coatomers causes membrane deformation and budding. Bilayer fusion at the base of a bud induced by fatty acyl-CoA results in vesicle release. Roles for PLD in both vesicle formation and fusion have been suggested. Removal of the coat, which is necessary for vesicle fusion at the target membrane, requires inactivation of ARF by hydrolysis of bound GTP to GDP.

[0013] Mammalian ARFs are divided into three classes based on size, amino acid sequence, gene structure, and phylogenetic analysis; ARF1, ARF2, and ARF3 are in class I, ARF4 and ARF5 are in class II, and ARF6 is in class III. Non-mammalian class I, II, and III ARFs have also been found. A role for class I ARFs 1 and 3 in ER to Golgi and intra-Golgi transport is well established (Cosson & Letourneur, 1997, *Curr. Opin. Cell Biol.* 9: 484-487, which is incorporated by reference herein in its entirety). ARF6 has been implicated in a pathway involving plasma membrane and a tubulovesicular compartment that is distinct from previously characterized endosomes (Radhakrishna & Donaldson, 1997, *J Cell Biol* 139: 49-61; Moss & Vaughn, 1998, *Jnl Biol Chem* 273: 21431-21434, both of which are incorporated by reference herein in their entireties).

[0014] An ARF1 GAP (purified and cloned from liver) was recruited to membranes by overexpression of ERD2, a membrane receptor that recognizes the C-terminal sequence (Lys-Asp-Glu-Leu) found on certain soluble proteins (KDEL proteins) of the endoplasmic reticulum and serves to retrieve them if they are transported to the Golgi (Aoe *et al.*, 1997, *EMBO J.* 16: 7305-7316, which is incorporated by reference herein in its entirety). Oligomerized ERD2 associated with the GAP, which then inactivated membrane-bound ARF and produced in the transfected cells a phenotype like that resulting from inhibition of ARF guanine-nucleotide exchange proteins (GEPs). It was later shown that overexpression of lysozyme with a KDEL terminus, which was intended to increase engagement of the KDEL receptor in retrograde retrieval transport, increased its interaction with ARF GAP and ARF inactivation, demonstrating a way in which vesicle content/cargo can influence a transport pathway (Cosson & Letourneur, 1997, *Curr. Opin. Cell Biol.* 9: 484-487, which is incorporated by reference herein in its entirety).

[0015] ARF GAP activity appears to be modulated by phospholipids. GAP activity is strongly stimulated by PIP_2 and was inhibited by phosphatidylcholine, as indicated by Makler *et al* (1995, *Jnl Biol Chem* 270: 5232-5237, which is incorporated by reference herein in its entirety) using both crude and purified GAP preparations. The effects of phospholipids on the ARF GAP may be related to a recently discovered role of ARF in the regulation of phospholipid metabolism (Kahn *et al.*, 1993, *Cell* 75: 1045-1048, which is incorporated by reference herein in its entirety), where ARF was identified as the cytosolic GTP binding protein that activates phospholipase D. Activated phospholipase D cleaves phosphatidylcholine to produce phosphatidic acid and choline. A feedback loop mechanism has been proposed where following the activation of phospholipase D by GTP-bound ARF, an increase in local phosphatidic acid concentration (and possibly also a decrease in phosphatidylcholine concentration) brings about an increase in the activity of the ARF GAP, resulting in the hydrolysis of ARF-bound GTP and the cessation of phospholipase D activity.

[0016] In other experiments using recombinant GAP, dioleylglycerol dramatically increased the activity of the recombinant GAP (amino acids 1-257). Because monosaturated diacylglycerols are produced chiefly from PC via the sequential action of PLD and phosphatidate phosphohydrolase (whereas polyunsaturated diacylglycerols are derived from PIP_2 via phosphatidylinositol phospholipase C action), it was suggested that PLD activity could be a major regulator of ARF GAP (Antonny *et al.*, 1997, *J Biol Chem* 272: 30848-30851, which is incorporated by reference herein in its entirety). GAP activity was varied 100-fold by altering relative amounts of PC and diacylglycerol (Antonny *et al.*, 1997, *supra*, which is incorporated by reference herein in its entirety), and similar effects were observed on the activity of and lipid binding by Gcs1, an analogous ARF GAP from yeast.

[0017] By comparing systematically the effects of phospholipid polar head groups and hydrocarbon chains on binding to the two GAPs, it was concluded that membrane association depended chiefly on hydrophobic interaction of the protein with hydrocarbon moieties of the lipid, which is favored by small head groups, and the conformation of monounsaturated acyl chains (Antonnye *et al.*, 1997, *supra*, which is incorporated by reference herein in its entirety). In this view, the activation of ARF GAP results from increasing its concentration at the membrane where ARF-GTP resides. ARF activation of PLD leading to decreased PC and increased diacylglycerol levels would promote

translocation of ARF GAP to a vesicle membrane where it could inactivate ARF-GTP and thereby terminate PLD action. The ARF GAPs that are activated by PIP_2 or other phosphoinositides are presumably subject to different kinds of regulation.

[0018] The process of abscission in plants affects many important physiological events in the various stages of plant growth. Thus, the ability to control abscission would be beneficial for many aspects of plant biology and crop science. What is needed in the art is a method of genetically modulating the process of organ abscission in plants.

Summary of the Invention

[0019] In one aspect of the invention, a plant exhibiting decreased organ abscission is provided. The plant has a nucleotide sequence with a modified ARF GAP domain. The decreased organ abscission may be include floral abscission. In some embodiments, the organ abscission may be abolished. In some embodiments, the plant may be *Arabidopsis thaliana*. In some embodiments of the invention, the nucleotide sequence can be SEQ ID NO: 3 or SEQ ID NO: 5.

[0020] Aspects of the invention also include the sequence of SEQ ID NO: 3 and SEQ ID NO:5, or isolated nucleotide sequences that hybridize to the complement of the sequence of SEQ ID NO: 3 or SEQ ID NO:5 under moderate stringency, where expression of the nucleotide sequence in a plant results in reduced or abolished abscission.

[0021] A further aspect of the invention includes a method of preventing organ loss in a plant, by mutating the ARF GAP domain of a gene in a plant and determining if the mutation results in the prevention of organ loss in the plant. In some embodiments, the organ loss can be floral organ loss. In some embodiments, the mutation is performed by exposure to ethyl methanesulphonate (EMS). In some embodiments, the gene can be the nucleotide sequence of SEQ ID NO: 1.

[0022] Additional embodiments of the invention provide an isolated polypeptide comprising the amino acid sequence of SEQ ID NO: 4 or SEQ ID NO: 6.

Brief Description of the Drawings

[0023] **Figure 1.** Location of *NEVERSHED* genomic sequence on chromosome 5. The location of markers CER970 in the region corresponding to bacterial artificial chromosome (BAC) MDK4 and RPK in the region corresponding to BAC GA469 are

indicated. The location of exons within the *NEVERSHED* genomic sequence and the *nev-2* mutation are indicated.

[0024] **Figure 2.** Alignment of putative ARF GAP domain from the *NEVERSHED* amino acid sequence with ARF GAP domains from rat ARFGAP1, yeast GCS1, fly CG8243 and worm W09D10.1. Identical amino acids for all sequences are indicated below the alignment. C to Y substitution caused by *nev-1* mutation is indicated.

Detailed Description of the Preferred Embodiment

[0025] Embodiments of the invention relate to the discovery that genetic modification of particular genes in a plant can result in prevention of organ abscission. In one embodiment, modification of the ARF GAP domain of a gene resulted in prevention of flower abscission. Accordingly, one embodiment of the invention is a method for preventing organ abscission in a plant by modifying the ARF GAP domain of gene. In another embodiment, the invention includes methods of preventing floral abscission in a plant by modifying the ARF GAP domain of a gene.

[0026] Particularly relevant genes include the family of *NEVERSHED* genes and mutations thereof which, as discussed below, have been discovered in *Arabidobsis*. However, these methods are not limited to any particular plant type. It is expected that similar mutations in other plants will result in similar phenotypes.

[0027] It was discovered that mutating the ARF GAP domain of genes within the NEVERSHED family resulted in plants having reduced, or abolished floral abscission. Thus, embodiments of the invention include methods of preventing floral abscission by mutating the *NEVERSHED* gene, and more specifically, mutating the ARF GAP domain of the *NEVERSHED* gene.

[0028] Other embodiments of the invention include plants that overexpress mutated forms of the *NEVERSHED* gene. Methods of transforming plants with the *NEVERSHED* gene are described below.

[0029] The present invention is based, in part, upon the identification, isolation, cloning and sequencing of a novel gene family regulating abscission in plants. By the present invention, we describe several novel family members, *NEVERSHED*, and homologues and mutants thereof, identified in *Arabidopsis thaliana* and other plants.

[0030] Thus, in one series of embodiments, the present invention provides isolated nucleic acids including nucleotide sequences comprising or derived from the *NEVERSHED* genes and/or encoding polypeptides comprising or derived from the NEVERSHED proteins. The *NEVERSHED* sequences of the invention include the specifically disclosed sequences, splice variants of these sequences, allelic variants of these sequences, synonymous sequences, and homologous or orthologous variants of these sequences. Thus, for example, the invention provides genomic and cDNA sequences from the *NEVERSHED* gene. The present invention also provides allelic variants and homologous or orthologous sequences by providing methods by which such variants may be routinely obtained. The present invention also specifically provides for mutant or variants of the *NEVERSHED* sequences by disclosing a number of specific mutant sequences and by providing methods by which other such variants may be routinely obtained. Because the nucleic acids of the invention may be used in a variety of applications, various subsets of the *NEVERSHED* sequences and combinations of the *NEVERSHED* sequences with heterologous sequences are also provided, particularly the ARF domain. For example, for use in allele specific hybridization screening or PCR amplification techniques, subsets of the *NEVERSHED* sequences, including both sense and antisense sequences, and both normal and mutant sequences, as well as intronic, exonic and untranslated sequences, are provided. Such sequences may comprise a small number of consecutive nucleotides from the sequences which are disclosed or otherwise enabled herein but preferably include at least 8-10, and more preferably 9-25, consecutive nucleotides from a *NEVERSHED* sequence. Such sequences are particularly useful to identify modulators of *NEVERSHED*, including inhibitors and inducers of *NEVERSHED*, from, for example, crystal structures of the ARF domain. Other preferred subsets of the *NEVERSHED* sequences include those encoding one or more of the functional domains or antigenic determinants of the NEVERSHED proteins and, in particular, may include either normal (wild-type) or mutant sequences, particularly the ARF domain. The invention also provides for various nucleic acid constructs in which *NEVERSHED* sequences, either complete or subsets, are operably joined to exogenous sequences to form cloning vectors, expression vectors, fusion vectors, transgenic constructs, and the like. Thus, in accordance with another aspect of the invention, a recombinant vector for transforming a *NEVERSHED* sequence to cells is provided.

[0031] Embodiments of the invention also include several homologs of an *Arabidopsis thaliana NEVERSHED* gene (SEQ ID NOs 1 and 2), and mutants thereof that affect abscission. These homologs include the MKP6.22 homolog (SEQ ID NOs 9 and 10), the F13M22.5 homolog (SEQ ID NOs 11 and 12), the F17A17.28 homolog (SEQ ID NOs 13 and 14), the F5K20.10 homolog (SEQ ID NOs 15 and 16), and the MZA15.17 homolog (SEQ ID NOs 17 and 18).

[0032] Accordingly, in another series of embodiments, the present invention provides methods of screening or identifying proteins, small molecules or other compounds which are capable of inducing or inhibiting the expression of the *NEVERSHED* genes and proteins. The assays may be performed *in vitro* using transformed or non-transformed cells, immortalized cell lines, or *in vivo* using transformed plant models enabled herein. In particular, the assays may detect the presence of increased or decreased expression of *NEVERSHED* (from *Arabidopsis* or other plants) genes or proteins on the basis of increased or decreased mRNA expression, increased or decreased levels of NEVERSHED protein products, or increased or decreased levels of expression of a marker gene (*e.g.*, beta-galactosidase, green fluorescent protein, alkaline phosphatase or luciferase) operably joined to a *NEVERSHED* 5' regulatory region in a recombinant construct. Cells known to express a particular *NEVERSHED* sequence, or transformed to express a particular *NEVERSHED* sequence, are incubated and one or more test compounds are added to the medium. After allowing a sufficient period of time (*e.g.*, 0-72 hours) for the compound to induce or inhibit the expression of the *NEVERSHED* sequence, any change in levels of expression from an established baseline may be detected using any of the techniques described above.

[0033] In another series of embodiments, the present invention provides methods for identifying proteins and other compounds which bind to, or otherwise directly interact with, the *NEVERSHED* gene or the NEVERSHED protein. The proteins and compounds will include endogenous cellular components which interact with *NEVERSHED in vivo* and which, therefore, provide new targets for agricultural products, as well as recombinant, synthetic and otherwise exogenous compounds which may have *NEVERSHED* binding capacity and, therefore, may be candidates for defoliates. Thus, in one series of embodiments, HTS protein or DNA chips, cell lysates or tissue homogenates may be screened for proteins or other compounds which bind to one of the normal or mutant

NEVERSHED genes. Alternatively, any of a variety of exogenous compounds, both naturally occurring and/or synthetic (*e.g.*, libraries of small molecules or peptides), may be screened for *NEVERSHED* binding capacity.

[0034] In each of these embodiments, an assay is conducted to detect binding between NEVERSHED protein and some other moiety. The NEVERSHED in these assays may be any polypeptide comprising or derived from a normal or mutant NEVERSHED protein, including functional domains or antigenic determinants of the NEVERSHED fusion proteins, such as the ARF domain. Binding may be detected by non-specific measures (*e.g.*, transcription modulation, altered chromatin structure, peptide production or changes in the expression of other downstream genes which can be monitored by differential display, 2D gel electrophoresis, differential hybridization, or SAGE methods) or by direct measures such as immunoprecipitation, the Biomolecular Interaction Assay (BIAcore) or alteration of protein gel electrophoresis. The preferred methods involve variations on the following techniques: (1) direct extraction by affinity chromatography; (2) co-isolation of NEVERSHED components and bound proteins or other compounds by immunoprecipitation; (3) BIAcore analysis; and (4) the yeast two-hybrid systems.

[0035] In another series of embodiments, the present invention provides for methods of identifying proteins, small molecules and other compounds capable of modulating the activity of normal or mutant NEVERSHED. In a particular aspect of the present invention, there are provided methods for identifying compounds capable of modulating specifically the ARF domain, more specifically, the ARF domain from plants and not other organisms such as mammals. Using normal cells or plants, the transformed cells and plant models of the present invention, or cells obtained from subjects bearing normal or mutant *NEVERSHED* genes, the present invention provides methods of identifying such compounds on the basis of their ability to affect the expression of *NEVERSHED*, the activity of *NEVERSHED*, the activity of other *NEVERSHED*-regulated genes, the activity of proteins that interact with normal or mutant NEVERSHED proteins, the intracellular localization of the NEVERSHED protein, changes in transcription activity, differentiation of abscission zones, metabolic measures such as the partitioning of carbon or nitrogen or nutrients, the occurrence or rate of vesicular transport, the levels or pattern of ARF-GTP, the presence or levels of membrane bound NEVERSHED, or other biochemical, histological, or

physiological markers which distinguish cells bearing normal and modulated ARF activity in plants and in animals.

[0036] In accordance with another aspect of the invention, the proteins of the invention can be used as starting points for rational chemical design to provide ligands or other types of small chemical molecules. Alternatively, small molecules or other compounds identified by the above-described screening assays may serve as "lead compounds" in design of modulators of abscission in plants.

[0037] In order to understand genetic control of floral abscission, we developed mutants in which floral abscission was specifically and completely blocked. Genetic screens of EMS-mutagenized populations led to the identification and characterization of two independent alleles of a recessive *Arabidopsis* mutant in which floral organ abscission fails to occur throughout the lifetime of the plant. Because floral abscission, or organ shedding of the sepals, petals and stamens, does not take place in these mutants, the corresponding gene has been named *NEVERSHED*. Through characterization of an *Arabidopsis* mutant which fails to shed its floral organs, and by determining the molecular basis for these defects, abscission zone development can be examined and the cascades of gene activity that lead to the differentiation of this cell type can be determined.

[0038] As used herein, the term "substantially pure" as used herein refers to polypeptides which are substantially free of other proteins, lipids, carbohydrates or other materials with which it is naturally associated. One skilled in the art can purify a polypeptide using standard techniques for protein purification. The purity of a polypeptide can also be determined by amino-terminal amino acid sequence analysis.

[0039] Embodiments of the invention also include functional NEVERSHED polypeptides, and functional fragments thereof. As used herein, the term "functional polypeptide" refers to a polypeptide which possesses biological function or activity which is identified through a defined functional assay and which is associated with a particular biologic, morphologic, or phenotypic alteration in the cell. The term "functional fragments of NEVERSHED polypeptide", refers to all fragments of NEVERSHED that retain NEVERSHED activity, *e.g.*, preventing organ abscission. Biologically functional fragments, for example, can vary in size from a polypeptide fragment as small as an epitope capable of

binding an antibody molecule to a large polypeptide capable of participating in the characteristic induction or programming of phenotypic changes within a cell.

[0040] Many modifications of the NEVERSHED primary amino acid sequence may result in plants having reduced or abolished organ abscission. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All of the polypeptides produced by these modifications are included herein as long as the biological activity of NEVERSHED is present. Further, deletion of one or more amino acids can also result in a modification of the structure of the resultant molecule without significantly altering its activity. This can lead to the development of a smaller active molecule which could have broader utility. For example, it may be possible to remove amino or carboxy terminal amino acids required for NEVERSHED activity.

[0041] NEVERSHED polypeptides includes amino acid sequences substantially the same as the sequence set forth in SEQ ID NO:2, including mutants that result in plants having decreased organ abscission. The term "substantially the same" refers to amino acid sequences that retain the activity of NEVERSHED as described herein. The NEVERSHED polypeptides of the invention include conservative variations of the polypeptide sequence.

[0042] The term "conservative variation" as used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

[0043] NEVERSHED proteins can be analyzed by standard SDS-PAGE and/or immunoprecipitation analysis and/or Western blot analysis, for example. In addition, the *in vitro* synthesized (IVS) protein assay as described in the present examples can be used to analyze NEVERSHED protein product.

[0044] Embodiments of the invention also provide an isolated polynucleotide sequence encoding a polypeptide having the amino acid sequence of SEQ ID NO:2. The

term "isolated" as used herein includes polynucleotides substantially free of other nucleic acids, proteins, lipids, carbohydrates or other materials with which it is naturally associated. Polynucleotide sequences of the invention include DNA, cDNA and RNA sequences which encode NEVERSHED. It is understood that polynucleotides encoding all or varying portions of NEVERSHED are included herein, as long as they encode a polypeptide with NEVERSHED activity. Such polynucleotides include naturally occurring, synthetic, and intentionally manipulated polynucleotides as well as splice variants. For example, portions of the mRNA sequence may be altered due to alternate RNA splicing patterns or the use of alternate promoters for RNA transcription.

[0045] Moreover, *NEVERSHED* polynucleotides include polynucleotides having alterations in the nucleic acid sequence which still encode a polypeptide having the ability to prevent organ abscission. Alterations in *NEVERSHED* nucleic acids include but are not limited to intragenic mutations (*e.g.*, point mutation, nonsense (stop), antisense, splice site and frameshift) and heterozygous or homozygous deletions. Detection of such alterations can be done by standard methods known to those of skill in the art including sequence analysis, Southern blot analysis, PCR based analyses (*e.g.*, multiplex PCR, sequence tagged sites (STSs)) and *in situ* hybridization. Embodiments of the invention also include anti-sense polynucleotide sequences.

[0046] The polynucleotides described herein include sequences that are degenerate as a result of the genetic code. There are 20 natural amino acids, most of which are specified by more than one codon. Therefore, all degenerate nucleotide sequences are included in the invention as long as the amino acid sequence of NEVERSHED polypeptide encoded by such nucleotide sequences retains NEVERSHED activity. A "functional polynucleotide" denotes a polynucleotide which encodes a functional polypeptide as described herein. In addition, embodiments of the invention also include a polynucleotide encoding a polypeptide having the biological activity of an amino acid sequence of SEQ ID NO:2 and having at least one epitope for an antibody immunoreactive with NEVERSHED polypeptide.

[0047] As used herein, the terms "polynucleotides" and "nucleic acid sequences" refer to DNA, RNA and cDNA sequences.

[0048] The polynucleotide encoding NEVERSHED includes the nucleotide sequence in SEQ ID NO:1, as well as nucleic acid sequences complementary to that sequence. A complementary sequence may include an antisense nucleotide. When the sequence is RNA, the deoxyribonucleotides A, G, C, and T of SEQ ID NO:1 are replaced by ribonucleotides A, G, C, and U, respectively. Also included in the invention are fragments ("probes") of the above-described nucleic acid sequences that are at least 15 bases in length, which is sufficient to permit the probe to selectively hybridize to DNA that encodes the protein of SEQ ID NO: 1.

[0049] "Hybridization" refers to the process by which a nucleic acid strand joins with a complementary strand through base pairing. Hybridization reactions can be sensitive and selective so that a particular sequence of interest can be identified even in samples in which it is present at low concentrations. Suitably stringent conditions can be defined by, for example, the concentrations of salt or formamide in the prehybridization and hybridization solutions, or by the hybridization temperature, and are well known in the art. In particular, stringency can be increased by reducing the concentration of salt, increasing the concentration of formamide, or raising the hybridization temperature.

[0050] For example, hybridization under high stringency conditions could occur in about 50% formamide at about 37°C to 42°C. Hybridization could occur under reduced stringency conditions in about 35% to 25% formamide at about 30°C to 35°C. In particular, hybridization could occur under high stringency conditions at 42°C in 50% formamide, 5X SSPE, 0.3% SDS, and 200 n/ml sheared and denatured salmon sperm DNA. Hybridization could occur under reduced stringency conditions as described above, but in 35% formamide at a reduced temperature of 35°C. The temperature range corresponding to a particular level of stringency can be further narrowed by calculating the purine to pyrimidine ratio of the nucleic acid of interest and adjusting the temperature accordingly. Variations on the above ranges and conditions are well known in the art.

[0051] "Selective hybridization" as used herein refers to hybridization under moderately stringent or highly stringent physiological conditions (See, for example, the techniques described in Maniatis *et al.*, 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, N.Y., which is incorporated by reference herein in its entirety), which distinguishes related from unrelated NEVERSHED nucleotide sequences.

[0052] Another aspect of the invention is polypeptides or fragments thereof which have at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more than about 95% homology to one of the polypeptides of SEQ ID NO:2, and sequences substantially identical thereto, or a fragment comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof. Homology may be determined using any of the methods described herein which align the polypeptides or fragments being compared and determines the extent of amino acid identity or similarity between them. It will be appreciated that amino acid "homology" includes conservative amino acid substitutions such as those described above.

[0053] The polypeptides or fragments having homology to one of the polypeptides of SEQ ID NO:2, and sequences substantially identical thereto, or a fragment comprising at least about 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof may be obtained by isolating the nucleic acids encoding them using the techniques described herein

[0054] Alternatively, the homologous polypeptides or fragments may be obtained through biochemical enrichment or purification procedures. The sequence of potentially homologous polypeptides or fragments may be determined by proteolytic digestion, gel electrophoresis and/or microsequencing. The sequence of the prospective homologous polypeptide or fragment can be compared to one of the polypeptides of SEQ ID NO:2, and sequences substantially identical thereto, or a fragment comprising at least about 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof using any of the programs described above.

[0055] Also included in embodiments of the invention are nucleotide sequences that are greater than 70% homologous with the sequence of SEQ ID NO: 1, but still retain the ability to decrease or prevent organ abscission. Other embodiments of the invention include nucleotide sequences that are greater than 75%, 80%, 85%, 90% or 95% homologous with the sequence of SEQ ID NO: 1, but still retain the ability to decrease or prevent organ or flower abscission.

[0056] Specifically disclosed herein is a genomic sequence for *NEVERSHED*. DNA sequences of the invention can be obtained by several methods. For example, the DNA can be isolated using hybridization or computer-based techniques which are well known in

the art. Such techniques include, but are not limited to: 1) hybridization of genomic or cDNA libraries with probes to detect homologous nucleotide sequences; 2) antibody screening of expression libraries to detect cloned DNA fragments with shared structural features; 3) polymerase chain reaction (PCR) on genomic DNA or cDNA using primers capable of annealing to the DNA sequence of interest; 4) computer searches of sequence databases for similar sequences; and 5) differential screening of a subtracted DNA library.

[0057] Screening procedures which rely on nucleic acid hybridization make it possible to isolate any gene sequence from any organism, provided the appropriate probe is available. Oligonucleotide probes, which correspond to a part of the *NEVERSHED* sequence encoding the protein in question, can be synthesized chemically. This requires that short, oligopeptide str*etc*hes of the amino acid sequence must be known. The DNA sequence encoding the protein can be deduced from the genetic code, however, the degeneracy of the code must be taken into account. It is possible to perform a mixed addition reaction when the sequence is degenerate. This includes a heterogeneous mixture of denatured double-stranded DNA. For such screening, hybridization is preferably performed on either single-stranded DNA or denatured double-stranded DNA. Hybridization is particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. In other words, by using stringent hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Wallace *et al.*, 1981, *Nucl. Acid Res.*, 9:879, which is incorporated by reference herein in its entirety). Alternatively, a subtractive library, as illustrated herein is useful for elimination of non-specific cDNA clones.

[0058] Among the standard procedures for isolating cDNA sequences of interest is the formation of plasmid- or phage-carrying cDNA libraries which are derived from reverse transcription of mRNA which is abundant in donor cells that have a high level of genetic expression. When used in combination with polymerase chain reaction technology, even rare expression products can be cloned. In those cases where significant portions of the amino acid sequence of the polypeptide are known, the production of labeled single or double-stranded DNA or RNA probe sequences duplicating a sequence putatively present in

the target cDNA may be employed in DNA/DNA hybridization procedures which are carried out on cloned copies of the cDNA which have been denatured into a single-stranded form (Jay *et al.*, 1983, *Nucl. Acid Res.*, 11:2325, which is incorporated by reference herein in its entirety).

[0059] A cDNA expression library, such as lambda gt11, can be screened indirectly for NEVERSHED peptides using antibodies specific for NEVERSHED. Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of *NEVERSHED* cDNA.

[0060] DNA sequences encoding NEVERSHED can be expressed *in vitro* by DNA transfer into a suitable host cell. "Host cells" are cells in which a vector can be propagated and its DNA expressed. The term also includes any progeny or graft material, for example, of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication. However, such progeny are included when the term "host cell" is used. Methods of stable transfer, meaning that the foreign DNA is continuously maintained in the host, are known in the art.

[0061] As part of the present invention, the *NEVERSHED* polynucleotide sequences may be inserted into a recombinant expression vector. The terms "recombinant expression vector" or "expression vector" refer to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of the *NEVERSHED* genetic sequence. Such expression vectors contain a promoter sequence which facilitates the efficient transcription of the inserted *NEVERSHED* sequence. The expression vector typically contains an origin of replication, a promoter, as well as specific genes which allow phenotypic selection of the transformed cells.

[0062] Methods which are well known to those skilled in the art can be used to construct expression vectors containing the *NEVERSHED* coding sequence and appropriate transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* recombination/genetic techniques.

[0063] A variety of host-expression vector systems may be utilized to express the *NEVERSHED* coding sequence. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the *NEVERSHED* coding sequence; yeast transformed with

recombinant yeast expression vectors containing the *NEVERSHED* coding sequence; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing the *NEVERSHED* coding sequence; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the *NEVERSHED* coding sequence; or animal cell systems infected with recombinant virus expression vectors (*e.g.*, retroviruses, adenovirus, vaccinia virus) containing the *NEVERSHED* coding sequence, or transformed animal cell systems engineered for stable expression.

[0064] Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, transcription enhancer elements, transcription terminators, *etc.* may be used in the expression vector (see *e.g.*, Bitter *et al.*, 1987, *Methods in Enzymol* 153:516-544, which is incorporated by reference herein in its entirety). For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage gamma, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used. When cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the inserted *NEVERSHED* coding sequence.

[0065] Isolation and purification of recombinantly expressed polypeptide, or fragments thereof, may be carried out by conventional means including preparative chromatography and immunological separations involving monoclonal or polyclonal antibodies.

[0066] Aspects of the invention also include antibodies immunoreactive with NEVERSHED polypeptide or antigenic fragments thereof. Antibody which consists essentially of pooled monoclonal antibodies with different epitopic specificities, as well as distinct monoclonal antibody preparations are provided. Monoclonal antibodies are made from antigen containing fragments of the protein by methods well known to those skilled in

the art (Kohler, *et al.*, 1975, *Nature* 256:495, which is incorporated by reference herein in its entirety).

[0067] The term "antibody" as used in this invention includes intact molecules as well as fragments thereof, such as Fab, F(ab')2, and Fv which are capable of binding to an epitopic determinant present in NEVERSHED polypeptide. Such antibody fragments retain some ability to selectively bind with its antigen or receptor.

[0068] Methods of making these fragments are known in the art. (See for example, Harlow and Lane, 1988, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, which is incorporated by reference herein in its entirety).

[0069] As used in this invention, the term "epitope" refers to an antigenic determinant on an antigen to which the paratope of an antibody binds. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

[0070] Antibodies which bind to the NEVERSHED polypeptide of the invention can be prepared using an intact polypeptide or fragments containing small peptides of interest as the immunizing antigen. For example, it may be desirable to produce antibodies that specifically bind to the N- or C-terminal domains of NEVERSHED. The polypeptide or peptide used to immunize an animal which is derived from translated cDNA or chemically synthesized which can be conjugated to a carrier protein, if desired. Such commonly used carriers which are chemically coupled to the immunizing peptide include keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), and tetanus toxoid.

[0071] Polyclonal or monoclonal antibodies can be further purified, for example, by binding to and eluting from a matrix to which the polypeptide or a peptide to which the antibodies were raised is bound. Those of skill in the art will know of various techniques common in the immunology arts for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies. (See for example, Coligan *et al.*, 1994, *Unit 9, Current Protocols in Immunology*, Wiley Interscience, which is incorporated by reference herein in its entirety).

[0072] It is also possible to use the anti-idiotype technology to produce monoclonal antibodies which mimic an epitope. For example, an anti-idiotypic monoclonal

antibody made to a first monoclonal antibody will have a binding domain in the hypervariable region which is the "image" of the epitope bound by the first monoclonal antibody.

[0073] In another embodiment, the embodiments of the invention provide a method for producing a genetically modified plant characterized as having decreased or abolished abscission as compared to a plant which has not been genetically modified (*e.g.*, a wild-type plant). The method includes the steps of contacting a plant cell with at least one vector containing at least one nucleic acid sequence encoding a *NEVERSHED* mutant gene, wherein the nucleic acid sequence is operably associated with a promoter, to obtain a transformed plant cell; producing a plant from the transformed plant cell; and thereafter selecting a plant exhibiting reduced abscission. The resulting mutant phenotype will be known as the *nevershed* mutant.

[0074] The term "genetic modification" as used herein refers to the introduction of one or more heterologous nucleic acid sequences, *e.g.*, a *NEVERSHED* mutant gene, into one or more plant cells, which can generate whole, sexually competent, viable plants having the *nevershed* mutant phenotype. The term "genetically modified" as used herein refers to a plant which has been generated through the aforementioned process. Genetically modified plants of the invention are capable of self-pollinating or cross-pollinating with other plants of the same species so that the foreign gene, carried in the germ line, can be inserted into or bred into agriculturally useful plant varieties. The term "plant cell" as used herein refers to protoplasts, gamete producing cells, and cells which regenerate into whole plants. Accordingly, a seed comprising multiple plant cells capable of regenerating into a whole plant, is included in the definition of "plant cell".

[0075] As used herein, the term "plant" refers to either a whole plant, a plant part, a plant cell, or a group of plant cells, such as plant tissue, for example. Plantlets are also included within the meaning of "plant". Plants included in the invention are any plants amenable to transformation techniques, including angiosperms, gymnosperms, monocotyledons and dicotyledons.

[0076] Examples of monocotyledonous plants include, but are not limited to, asparagus, field and sweet corn, barley, wheat, rice, sorghum, onion, pearl millet, rye and oats. Examples of dicotyledonous plants include, but are not limited to tomato, tobacco,

cotton, rapeseed, field beans, soybeans, peppers, lettuce, peas, alfalfa, clover, cole crops or *Brassica oleracea* (*e.g.*, cabbage, broccoli, cauliflower, brussel sprouts), radish, carrot, beets, eggplant, spinach, cucumber, squash, melons, cantaloupe, sunflowers and various ornamentals. Woody species include poplar, pine, sequoia, cedar, oak, *etc.*

[0077] The term "heterologous nucleic acid sequence" as used herein refers to a nucleic acid foreign to the recipient plant host or, native to the host if the native nucleic acid is substantially modified from its original form. For example, the term includes a nucleic acid originating in the host species, where such sequence is operably linked to a promoter that differs from the natural or wild-type promoter. In one embodiment, at least one nucleic acid sequence encoding a *NEVERSHED* mutant is operably linked with a promoter. In another embodiment, a different gene having a mutated ARF GAP domain is operably linked with a promoter. It may be desirable to introduce more than one copy of a *NEVERSHED* mutant polynucleotide into a plant for enhanced expression. For example, multiple copies of the gene would have the effect of increasing production of the *NEVERSHED* mutant gene in the plant.

[0078] Genetically modified plants of the present invention are produced by contacting a plant cell with a vector including at least one nucleic acid sequence encoding a *NEVERSHED* mutant. To be effective once introduced into plant cells, the mutant *NEVERSHED* nucleic acid sequence must be operably associated with a promoter which is effective in the plant cells to cause transcription of *NEVERSHED* mutant. Additionally, a polyadenylation sequence or transcription control sequence, also recognized in plant cells may also be employed. It is preferred that the vector harboring the nucleic acid sequence to be inserted also contain one or more selectable marker genes so that the transformed cells can be selected from non-transformed cells in culture, as described herein.

[0079] The term "operably associated" refers to functional linkage between a promoter sequence and a nucleic acid sequence regulated by the promoter. The operably linked promoter controls the expression of the nucleic acid sequence.

[0080] The expression of structural genes may be driven by a number of promoters. Although the endogenous, or native promoter of a structural gene of interest may be utilized for transcriptional regulation of the gene, preferably, the promoter is a foreign regulatory sequence. For plant expression vectors, suitable viral promoters include the 35S

RNA and 19S RNA promoters of CaMV (Brisson, *et al.*, 1984, *Nature* 310:511; Odell, *et al.*, 1985, *Nature* 313:810, both of which are incorporated by reference herein in their entireties); the full-length transcript promoter from Figwort Mosaic Virus (FMV) (Gowda, *et al.*, 1989, *J. Cell Biochem.*, 13D: 301, which is incorporated by reference herein in its entirety) and the coat protein promoter to TMV (Takamatsu, *et al.*, 1987, *EMBO J.* 6:307, which is incorporated by reference herein in its entirety). Alternatively, plant promoters such as the light-inducible promoter from the small subunit of ribulose bis-phosphate carboxylase (ssRUBISCO) (Coruzzi, *et al.*, 1984, *EMBO J.*, 3:1671; Broglie, *et al.*, 1984, *Science* 224:838, both of which are incorporated by reference herein in their entireties); mannopine synthase promoter (Velten, *et al.*, 1984, *EMBO J.*, 3:2723, which is incorporated by reference herein in its entirety) nopaline synthase (NOS) and octopine synthase (OCS) promoters (carried on tumor-inducing plasmids of *Agrobacterium tumefaciens*) or heat shock promoters, *e.g.*, soybean hsp17.5-E or hsp17.3-B (Gurley, *et al.*, 1986, *Mol. Cell. Biol.* 6:559; Severin, *et al.*, 1990, *Plant Mol. Biol.* 15:827, both of which are incorporated by reference herein in their entireties) may be used.

[0081] Promoters useful in the invention include both natural constitutive and inducible promoters as well as engineered promoters. One embodiment of a promoter can be found in SEQ ID NO: 7, which includes the sequences upstream from SEQ ID NO:1. The CaMV promoters are examples of constitutive promoters. To be most useful, an inducible promoter should 1) provide low expression in the absence of the inducer; 2) provide high expression in the presence of the inducer; 3) use an induction scheme that does not interfere with the normal physiology of the plant; and 4) have no effect on the expression of other genes. Examples of inducible promoters useful in plants include those induced by chemical means, such as the yeast metallothionein promoter which is activated by copper ions (Mett, *et al.*, 1993, *Proc. Natl. Acad. Sci.* U.S.A. 90:4567, which is incorporated by reference herein in its entirety); In2-1 and In2-2 regulator sequences which are activated by substituted benzenesulfonamides, *e.g.*, herbicide safeners (Hershey, *et al.*, 1991, *Plant Mol. Biol.* 17:679, which is incorporated by reference herein in its entirety); and the GRE regulatory sequences which are induced by glucocorticoids (Schena, *et al.*, 1991, *Proc. Natl. Acad. Sci. U.S.A* 88:10421, which is incorporated by reference herein in its entirety). Other promoters, both constitutive and inducible will be known to those of skill in the art.

[0082] The particular promoter selected should be capable of causing sufficient expression to result in the production of an effective amount of structural gene product, *e.g.*, a *NEVERSHED* mutant, to cause increased yield and/or increased biomass. The promoters used in the vector constructs of the present invention may be modified, if desired, to affect their control characteristics.

[0083] Tissue specific promoters may also be utilized in the present invention. An example of a tissue specific promoter is the promoter active in shoot meristems (Atanassova, *et al.*, 1992, *Plant J.* 2:291, which is incorporated by reference herein in its entirety). Other tissue specific promoters useful in transgenic plants, including the cdc2a promoter and cyc07 promoter, will be known to those of skill in the art. (See for example, Ito, *et al.*, 1994, *Plant Mol. Biol.* 24:863; Martinez, *et al.*, 1992, *Proc. Natl. Acad. Sci. USA* 89:7360; Medford, *et al.*, 1991, *Plant Cell* 3:359; Terada, *et al.*, 1993, *Plant Journal* 3:241; Wissenbach, *et al.*, 1993, *Plant Journal* 4:411, all of which are incorporated by reference herein in their entireties).

[0084] Optionally, a selectable marker may be associated with the nucleic acid sequence to be inserted. As used herein, the term "marker" refers to a gene encoding a trait or a phenotype which permits the selection of, or the screening for, a plant or plant cell containing the marker. Preferably, the marker gene is an antibiotic resistance gene whereby the appropriate antibiotic can be used to select for transformed cells from among cells that are not transformed. Examples of suitable selectable markers include adenosine deaminase, dihydrofolate reductase, hygromycin-B-phospho-transferase, thymidine kinase, xanthine-guanine phospho-ribosyltransferase and amino-glycoside 3'-O-phospho-transferase II (kanamycin, neomycin and G418 resistance). Other suitable markers will be known to those of skill in the art.

[0085] Vector(s) employed in the present invention for transformation of a plant cell include a nucleic acid sequence encoding a NEVERSHED mutant, operably associated with a promoter. To commence a transformation process in accordance with the present invention, it is first necessary to construct a suitable vector and properly introduce it into the plant cell. Details of the construction of vectors utilized herein are known to those skilled in the art of plant genetic engineering.

[0086] *NEVERSHED* nucleic acid sequences utilized in the present invention can be introduced into plant cells using Ti plasmids of *Agrobacterium tumefaciens,* root-inducing (Ri) plasmids, and plant virus vectors. (For reviews of such techniques see, for example, Weissbach & Weissbach, 1988, *Methods for Plant Molecular Biology*, Academic Press, NY, Section VIII, pp. 421-463; and Grierson & Corey, 1988, *Plant Molecular Biology, 2d Ed.*, Blackie, London, Ch. 7-9, and Horsch, *et al.*, 1985, *Science* 227:1229, all of which are incorporated by reference herein in their entireties). In addition to plant transformation vectors derived from the Ti or root-inducing (Ri) plasmids of *Agrobacterium*, alternative methods may involve, for example, the use of liposomes, electroporation, chemicals that increase free DNA uptake, transformation using viruses or pollen and the use of microprojection.

[0087] One of skill in the art will be able to select an appropriate vector for introducing the NEVERSHED-encoding nucleic acid sequence in a relatively intact state. Thus, any vector which will produce a plant carrying the introduced DNA sequence should be sufficient. Even use of a naked piece of DNA would be expected to confer the properties of this invention, though at low efficiency. The selection of the vector, or whether to use a vector, is typically guided by the method of transformation selected.

[0088] The transformation of plants in accordance with the invention may be carried out in essentially any of the various ways known to those skilled in the art of plant molecular biology. (See, for example, *Methods of Enzymology*, Vol. 153, 1987, Wu and Grossman, Eds., Academic Press, which is incorporated by reference herein in its entirety). As used herein, the term "transformation" means alteration of the genotype of a host plant by the introduction of a *NEVERSHED* nucleic acid sequence or a *NEVERSHED* mutant nucleic acid sequence.

[0089] For example, a *NEVERSHED* nucleic acid sequence can be introduced into a plant cell utilizing *Agrobacterium tumefaciens* containing the Ti plasmid, as mentioned briefly above. In using an *A. tumefaciens* culture as a transformation vehicle, it is most advantageous to use a non-oncogenic strain of *Agrobacterium* as the vector carrier so that normal non-oncogenic differentiation of the transformed tissues is possible. It is also preferred that the *Agrobacterium* harbor a binary Ti plasmid system. Such a binary system comprises 1) a first Ti plasmid having a virulence region essential for the introduction of

transfer DNA (T-DNA) into plants, and 2) a chimeric plasmid. The latter contains at least one border region of the T-DNA region of a wild-type Ti plasmid flanking the nucleic acid to be transferred. Binary Ti plasmid systems have been shown effective to transform plant cells (De Framond, 1983, *Biotechnology* 1: 262; Hoekema, *et al.*, 1983, *Nature* 303:179, both of which are incorporated by reference herein in their entireties). Such a binary system is preferred because it does not require integration into the Ti plasmid of *Agrobacterium*, which is an older methodology.

[0090] Methods involving the use of *Agrobacterium* in transformation according to the present invention include, but are not limited to: 1) co-cultivation of *Agrobacterium* with cultured isolated protoplasts; 2) transformation of plant cells or tissues with Agrobacterium; or 3) transformation of seeds, apices or meristems with Agrobacterium. In addition, gene transfer can be accomplished by in planta transformation by Agrobacterium, as described by Bechtold, *et al.*, (C. R. Acad. Sci. Paris, 316:1194, 1993, which is incorporated by reference herein in its entirety) and exemplified in the Examples herein. This approach is based on the vacuum infiltration of a suspension of *Agrobacterium* cells.

[0091] One method of introducing NEVERSHED-encoding nucleic acid into plant cells is to infect such plant cells, an explant, a meristem or a seed, with transformed *Agrobacterium tumefaciens* as described above. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots, roots, and develop further into plants.

[0092] Alternatively, NEVERSHED encoding nucleic acid sequences can be introduced into a plant cell using mechanical or chemical means. For example, the nucleic acid can be mechanically transferred into the plant cell by microinjection using a micropipette. Alternatively, the nucleic acid may be transferred into the plant cell by using polyethylene glycol which forms a precipitation complex with genetic material that is taken up by the cell.

[0093] One or more *NEVERSHED* nucleic acid sequences can also be introduced into plant cells by electroporation (Fromm *et al.*, 1985, *Proc. Natl. Acad. Sci. U.S.A.* 82:5824, which is incorporated by reference herein in its entirety). In this technique, plant protoplasts are electroporated in the presence of vectors or nucleic acids containing the relevant nucleic acid sequences. Electrical impulses of high field strength reversibly

permeabilize membranes allowing the introduction of nucleic acids. Electroporated plant protoplasts reform the cell wall, divide and form a plant callus. Selection of the transformed plant cells with the transformed gene can be accomplished using phenotypic markers as described herein.

[0094] Another method for introducing one or more *NEVERSHED* nucleic acid sequences into a plant cell is high velocity ballistic penetration by small particles with the nucleic acid to be introduced contained either within the matrix of such particles, or on the surface thereof (Klein, *et al.*, 1987, *Nature* 327:70, which is incorporated by reference herein in its entirety). Bombardment transformation methods are also described in Sanford *et al.* (1991, *BioTechniques* 3:3-16) and Klein *et al.* (1992, *Bio/Techniques* 10:286), both of which are incorporated by reference herein in their entireties. Although typically only a single introduction of a new nucleic acid sequence is required, this method particularly provides for multiple introductions.

[0095] Cauliflower mosaic virus (CaMV) may also be used as a vector for introducing nucleic acid into plant cells (U.S. Pat. No. 4,407,956, which is incorporated by reference herein in its entirety). CaMV viral DNA genome is inserted into a parent bacterial plasmid creating a recombinant DNA molecule which can be propagated in bacteria. After cloning, the recombinant plasmid again may be cloned and further modified by introduction of the desired nucleic acid sequence. The modified viral portion of the recombinant plasmid is then excised from the parent bacterial plasmid, and used to inoculate the plant cells or plants.

[0096] As used herein, the term "contacting" refers to any means of introducing *NEVERSHED* into the plant cell, including chemical and physical means as described above. Preferably, contacting refers to introducing the nucleic acid or vector into plant cells (including an explant, a meristem or a seed), via *Agrobacterium tumefaciens* transformed with the NEVERSHED-encoding nucleic acid as described above.

[0097] Normally, a plant cell is regenerated to obtain a whole plant from the transformation process. The immediate product of the transformation is referred to as a "transgenote". The term "growing" or "regeneration" as used herein means growing a whole plant from a plant cell, a group of plant cells, a plant part (including seeds), or a plant piece (*e.g.*, from a protoplast, callus, or tissue part).

[0098] Regeneration from protoplasts varies from species to species of plants, but generally a suspension of protoplasts is first made. In certain species, embryo formation can then be induced from the protoplast suspension, to the stage of ripening and germination as natural embryos. The culture media will generally contain various amino acids and hormones, necessary for growth and regeneration. Examples of hormones utilized include auxins and cytokinins. It is sometimes advantageous to add glutamic acid and proline to the medium, especially for plant species such as corn and alfalfa. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these variables are controlled, regeneration is reproducible.

[0099] Regeneration also occurs from plant callus, explants, organs or parts. Transformation can be performed in the context of organ or plant part regeneration. (See *Methods in Enzymology*, Vol. 118 and Klee, *et al.*, 1987, *Annual Review of Plant Physiology*, 38:467, which is incorporated by reference herein in its entirety). Utilizing the leaf disk-transformation-regeneration method of Horsch, *et al.* (1985, *Science* 227:1229, which is incorporated by reference herein in its entirety), disks are cultured on selective media, followed by shoot formation in about 2-4 weeks. Shoots that develop are excised from calli and transplanted to appropriate root-inducing selective medium. Rooted plantlets are transplanted to soil as soon as possible after roots appear. The plantlets can be repotted as required, until reaching maturity.

[0100] In vegetatively propagated crops, the mature transgenic plants are propagated by utilizing cuttings or tissue culture techniques to produce multiple identical plants. Selection of desirable transgenotes is made and new varieties are obtained and propagated vegetatively for commercial use.

[0101] In seed propagated crops, the mature transgenic plants can be self crossed to produce a homozygous inbred plant. The resulting inbred plant produces seed containing the newly introduced foreign gene(s). These seeds can be grown to produce plants that would produce the selected phenotype, *e.g.* increased yield.

[0102] Parts obtained from regenerated plant, such as flowers, seeds, leaves, branches, roots, fruit, and the like are included in the invention, provided that these parts comprise cells that have been transformed as described. Progeny and variants, and mutants

of the regenerated plants are also included within the scope of the invention, provided that these parts comprise the introduced nucleic acid sequences.

[0103] Plants exhibiting decreased or abolished abscission as compared with wild-type plants can be selected by visual observation. The invention includes plants produced by the method of the invention, as well as plant tissue and seeds.

[0104] In yet another embodiment, the invention provides a method for producing a genetically modified plant cell such that a plant produced from said cell has decreased or abolished abscission as compared with a wild-type plant. The method includes contacting the plant cell with a *NEVERSHED* nucleic acid sequence to obtain a transformed plant cell; growing the transformed plant cell under plant forming conditions to obtain a plant having increased yield. Conditions such as environmental and promoter inducing conditions vary from species to species, but should be the same within a species.

[0105] In another embodiment, the invention provides a method of producing a plant having decreased abscission by contacting a susceptible plant with a *NEVERSHED* promoter-inducing amount of an agent which induces *NEVERSHED* gene expression, wherein induction of *NEVERSHED* gene expression results in production of a plant having decreased abscission as compared to a plant not contacted with the agent.

[0106] A "susceptible plant" refers to a plant that can be induced to utilize its endogenous *NEVERSHED* gene to achieve decreased abscission. The term "promoter inducing amount" refers to that amount of an agent necessary to elevate *NEVERSHED* gene expression above *NEVERSHED* expression in a plant cell not contacted with the agent. For example, a transcription factor or a chemical agent may be used to elevate gene expression from *NEVERSHED* native promoter, thus inducing the promoter and *NEVERSHED* gene expression.

[0107] The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only and are not intended to limit the scope of the invention.

EXAMPLES

Example 1

Isolation and identification of the *nevershed* mutant in *Arabidopsis*

[0108] Seeds of *Arabidopsis thaliana* ecotype Landsberg erecta (Ler) were mutagenized by exposure to ethyl methanesulphonate (EMS) using standard techniques, for example as described in Lilgejren *et al.* (2000, *Nature* 404: 766-770, which is incorporated by reference herein in its entirety). EMS-exposed seeds were germinated, and plants were screened for unusual phenotypes. As a result of two unrelated EMS screens, two *Arabidopsis* mutants were identified having a phenotype in which floral organ abscission, more particularly organ shedding of the sepals, petals and stamens, failed to occur throughout the lifetime of the plant. This mutant phenotype was called *nevershed* because floral abscission, or organ shedding of sepals, petals and stamens, did not take place in this mutant. The two EMS mutants were called *nev-1* and *nev-2*.

[0109] It was then determined that these mutants having the same phenotype were independent alleles the same locus, termed herein "*nev*", and represented a recessive *Arabidopsis* mutant termed herein "*nevershed*". The corresponding gene has been called *NEVERSHED*.

Example 2

Cloning of the *NEVERSHED* gene and mutant alleles

[0110] Using a standard map-based cloning approach (Konieczny and Ausubel, 1993, *Plant J* 4: 403-410, which is incorporated by reference herein in its entirety), the *nev* locus was mapped to chromosome 5 as follows. *nev-1* (SEQ ID NO: 3) and *nev-2* (SEQ ID NO: 5) mutants, in the Landsberg erecta (Ler) ecotype, were crossed to wild-type plants of the Columbia (Col) ecotype. After scoring the *nevershed* phenotype in the F2 generation, CAPS markers were used to map the mutation to chromosome 5 (Konieczny and Ausubel, 1993, *supra*, which is incorporated by reference herein in its entirety). By using additional CAPS markers on chromosome 5, the *nev* locus was mapped to an interval of ~37.5 kb between the CER970 and RPK markers the on bacterial artificial chromosomes (BACs) MDK4 (GenBank Accession No. AB010695) and GA469 (GenBank Accession No.

AP000380), respectively. Figure 1 shows the structure of the relevant region of chromosome 5. The genomic sequence can be found in SEQ ID NO: 8.

[0111] The open reading frames of 8 of the 11 annotated genes in this interval were sequenced. It was discovered that the *nev-2* mutant contained a single nucleotide mutation (G to A) in the 7th exon of the gene annotated as MDK4.13. Analysis of this DNA sequence indicates that the *nev-2* mutation would result in a truncated protein of 197 amino acids (SEQ ID NO: 6), as the nucleotide substitution from C to T present at position 592 of SEQ ID NO. 5 (the *nev-2* coding sequence) caused a glutamine codon (CAG) to be altered to a stop codon (TAG). The *nev-2* mutation fell within the ARF GAP domain of the NEVERSHED gene (Figure 1).

[0112] Upon sequencing the MDK4.13 open reading frame from the *nev-1* mutant, a single nucleotide mutation (C to T) was found in the 3rd exon of the gene annotated as MDK4.13. This resulted in a G to A modification at position 152 of SEQ ID NO. 3 (the *nev-1* coding sequence), altering a cysteine codon to a tyrosine codon. The resulting protein sequence (SEQ ID NO. 4) therefore has the amino acid tyrosine at position 51 rather than the amino acid cysteine. This particular cysteine is one of 4 cysteines that constitute a conserved zinc finger motif in the predicted ADP-Ribosylation Factor GTPase Activating Protein (ARF GAP) domain of the protein corresponding to MDK4.13. The *nev-1* mutation fell within the ARF GAP domain of the *NEVERSHED* gene (Figure 2).

[0113] The gene annotated as MDK4.13 is a predicted gene found in the *Arabidopsis* BAC known as MDK4 (GenBank Accession No. AB010695), and the 11 predicted exons of MDK4.13 located between positions 60024 and 63828 on BAC MDK4 can be joined to produce a protein annotated as BAB10754.1 (SEQ ID NO: 2) The identification of two independent mutations in this gene in both of the two nevershed mutants strongly suggests that the gene annotated as MDK4.13 is responsible for the discovered phenotypes. It follows, therefore, that the protein encoded by coding sequences of MKD4.13 is the NEVERSHED protein, and mutations of this protein result in a decrease or loss of organ/floral abscission.

[0114] Comparing the amino acid sequence of the *NEVERSHED* gene with other protein sequences indicates a predicted ADP-Ribosylation Factor GTPase Activating Protein (ARF GAP) domain having homology to ARF GAP proteins from a wide taxonomic

distribution (Figure 2). Structurally diverse GAPs are associated with all families of small GTP binding proteins, acting as signal terminators and possibly also in some cases as effectors downstream of the GTP binding protein (Boguski & McCormick 1993 *Nature* 366: 643-653, which is incorporated by reference herein in its entirety). ADP-ribosylation factors (ARFs) are 20-kDa guanine nucleotide-binding proteins, members of the Ras GTPase superfamily that were initially recognized and purified because of their ability to stimulate the ADP-ribosyltransferase activity of the cholera toxin A subunit. Like other GTP binding proteins, ARF becomes activated upon the binding of GTP, whereas GTP hydrolysis acts as a turn-off signal. The fact that purified ARF proteins have negligible GTPase activity has suggested that GTP hydrolysis by ARFs is dependent on a GTPase-activating protein (GAP). ARFs are critical components of several different vesicular trafficking pathways in all eukaryotic cells and activators of specific phospholipase Ds (PLDs).

Example 3

Expression of *NEVERSHED* and *NEVERSHED*-regulated genes

[0115] In one embodiment, RNA blot and *in situ* hybridization analyses using standard procedures, are carried out to determine the temporal and spatial expression profile of *NEVERSHED*, preferably using *Arabidopsis* plants transformed with an expression vector containing the *NEVERSHED* promoter fused to the β-glucuronidase reporter gene. In other embodiments, expression of *NEVERSHED* and/or *NEVERSHED*-regulated genes are carried out under a variety of conditions to characterize the function of the *NEVERSHED* gene and the NEVERSHED protein. One of skill of the art can design further studies of *NEVERSHED* and/or *NEVERSHED*-regulated genes tailored according to sequence-based homology predictions and results from other studies of *NEVERSHED* function.

[0116] *NEVERSHED* and various *nevershed* mutants provide tools for studying the genetic pathways involved in *Arabidopsis* abscission zone development. These tools can be used to determine the genes involved in abscission zone development in other plant species. In particular, the identification of genes that act upstream and downstream of *NEVERSHED* may indicate the genes and pathways involved in regulating the development of absicssion zones in plants. The discovery that *NEVERSHED* has a domain with strong

homology to ARF GAP indicates a potential mechanism by which *NEVERSHED* exerts control over the development of abscission zones.

Example 4

Expression of *NEVERSHED*: microarray analysis and expression profiling

[0117] Both microarray analyses and the existence of numerous enhancer-trap lines with abscission zone expression profiles will allow extensive investigations of genes involved in abscission development. Collections of T-DNA tagged lines generated over the last few years, with some having more than 150,000 individual lines, permit the rapid mutation of known genes. Accordingly, studying abscission zone development in *Arabidopsis* permits a rapid translation of gene discoveries into agricultural application.

[0118] Microarray approaches using DNA chips to monitor global changes in RNA expression provide the ability to detect numerous genes affected by a particular mutation (Schena *et al.*, 1995, *Science* 270: 467-469, which is incorporated by reference herein in its entirety), such as the *nevershed* mutation. In one embodiment, expression profiling is carried out using high-density chips developed through a consortium of plant scientists and Affymetrix Corporation to develop high-density chips containing most, or all, *Arabidopsis* genes. The Affymetrix chip design differs from the glass slide microarray systems currently available to the *Arabidopsis* community, in that gene coding regions on the Affymetrix chip are represented on average by about 20 oligonucleotides each, instead of being represented by cDNAs.

[0119] Because the oligonucleotides on the chip are short (25mers), they represent an improvement for monitoring the expression levels of genes which have close family members. In one embodiment, the low variability and high chip to chip reproducibility allows use of a single probe for hybridization rather than the two-color fluorescence system used with glass microarrays, further allowing direct comparisons of hybridization results between different experiments and different labs.

[0120] In one embodiment, probes for chip hybridization are prepared as follows: poly A^+-RNAs is isolated from the floral abscission zone regions of *nevershed* mutants and of wildtype plants using standard procedures (Carninci *et al.*, 1996, *Plant J* 17: 699-707; Theologis *et al.*, 1985, *J Mol Biol* 183: 53-68, both of which are incorporated by reference

herein in their entireties) at a range of affected stages determined through the mutant characterization described above. RNA probes are biotinylated, the probes are incubated with chips under conditons favorable for hybridization, and the array is stained with a phycoerythrin-streptavadin conjugate (Winzler *et al.*, 1998, *Science* 281: 1191-1197, which is incorporated by reference herein in its entirety). Arrays are then scanned with a laser confocal scanning device that detects and records the amount of fluorescence (Wodicka *et al.*, 1997, *Nature Biotechnology* 15: 1359-1367, which is incorporated by reference herein in its entirety).

[0121] Current software tools and database support available for expression data analysis include data files containing a list of array locations and associated intensities are generated first. These files are then entered into a relational database (Sybase), which allows reporting of experimental conditions and mapping of the array locations to those described by the chip manufacturer. Another relational database (RAD) is used to store expression data. RAD has tables organized in three categories 1) an array-specific set of tables specifying what is located where in the array, 2) tables containing experimental details, and 3) tables representing RNA abundance data. Data analysis using RAD tables is being improved through links to DOTS (database of transcribed sequences), as DOT allows transcribed sequences to be compared and likely sequence functions described. DOT links are the primary tools for gene discovery, as clones represented in arrays have little associated information other than sequence and its cDNA library/tissue source.

[0122] Based on the high frequency of abscission zone markers observed among enhancer trap lines (Campisi *et al.*, 1999, *Plant J* 17 699-707, which is incorporated by reference herein in its entirety), it is predicted that many genes-- as many as 4000 or more-- are expressed in abscission zones. Preferably, the Affymetrix chip is used for analysis of abscission zone markers, as this chip permits expression analysis of sufficiently large numbers of genes, is highly reproducible, and has hybridization conditions that allow for greatly increased sensitivity to detect relative RNA levels.

[0123] In other embodiments, a filtering strategy is used to characterize abscission zone markers of potential importance. Preferably, poly A^+-RNA is isolated from the abscission zone regions of *nevershed* and wild-type, respectively, and labeled for use in two separate microarrays; poly A^+-RNA from the abscission zone regions of the ethylene-

insensitive *ein2* mutants is isolated and labeled for use in a third microarray. Because abscission is delayed in the *ein2* mutant, but occurs eventually, the early events of abscission zone differentiation can be presumed to be unaffected in *ein2*.

[0124] If the same range of floral stages are reflected in the *ein2* RNA probes, then identifying clones which are dramatically affected in *nevershed* flowers but not in *ein2* flowers or wildtype, should identify the clones corresponding to genes involved in the earliest events of abscission zone differentiation. Clones in this "early" category are analyzed further by RNA blot analysis to: a) verify that expression is significantly altered; b) determine their temporal profile within floral abscission zones; and c) ascertain whether they are abscission-zone specific. Clones showing dramatic alteration in expression, which show the earliest temporal profiles, and which appear to be abscission-zone specific will be further analyzed as candidates to screen for corresponding loss-of-function mutants in T-DNA insertional collections.

[0125] In other embodiments, molecular markers from gene- and enhancer-trap collections, currently among the most informative tools available to characterize *Arabidopsis* mutants (Sundaresan *et al.*, 1995, *Genes Dev* 9: 1979-1810, which is incorporated by reference herein in its entirety) are used in crosses with developmental mutants to detect evidence of altered molecular differentiation even before phenotypic defects become visible.

[0126] Although abscission zone molecular markers are plentiful (Campisi *et al.*, 1999, *supra*, which is incorporated by reference herein in its entirety), their usefulness has not yet been exploited to study mutants in which abscission is disrupted or to uncover additional genes whose products are involved in the abscission process. In one study of over 11,300 enhancer trap lines that were stained and analyzed (Campisi *et al.*, 1999, *supra*, which is incorporated by reference herein in its entirety), about 16% or 1800 lines showed staining patterns in the abscission zone. These lines represent an invaluable resource to analyze the *nevershed* mutant as well as other mutants which disrupt the abscission process, as markers for numerous stages of abscission zone differentiation should be represented within this collection.

[0127] In one embodiment of the invention, abscission zone markers representing different temporal profiles are crossed with *nevershed* mutants in order to more precisely determine the stage at which abscission zone development is first affected in these mutants.

A collection of frozen, individually stained lines, will be screened for potential abscission zone markers, and seeds having potential abscission zone markers are obtained. Markers are selected from the collection to reflect as many temporal profiles as possible, including markers which are expressed specifically at the bases of developing floral organs as early as stage 6, and markers expressed as late as stage 17 in abscission zone scar tissue. Further embodiments include studies of the expression profiles of chitinase::GUS and glucanase::GUS abscission zone markers in *nevershed* mutant flowers.

Example 5

ARF GAP and vesicle transport in plant floral organ abscission

[0128] The present invention also includes wild-type and mutant ARF GAP polynucleotides and polypeptides that can be used to determine the role of vesicle transport in abscission zone differentiation and floral organ abscission. Moreover, the ability to genetically manipulate abscission zone differentiation in agronomically important plants will provide valuable opportunities to improve crop yield and to simplify harvesting.

[0129] For instance, temporarily unfavorable environmental conditions such as high temperatures, drought, and flooding cause flower bud abscission in important crops such as cotton and field beans (Lloyd, 1920, *Ann NY Acad Sci* 29:1; Osborne, 1989, *Crit Rev Plant Sci* 8: 103-129, both of which are incorporated by reference herein in their entireties). Environmental stresses including cold temperatures can cause flower bud abscission in fruit trees and other flowering plants, reducing the potential number of sites at which fruit can set and thereby reducing yield potential even if environmental conditions improve later in the season. If these crops are genetically engineered to delay abscission zone differentiation until after flower opening, crop yield will be significantly improved, particularly during periods of sudden, extreme weather conditions. Preventing development of particular abscission zones, such as the pedicel abscission zone, are anticipated to streamline mechanical harvesting of many fruit crops, as has already been demonstrated by the widespread agricultural use of *jointless* tomato crops (Osborne, 1989, *supra*; Szymkowiak and Irish, 1999, *Plant Cell* 11: 159-176, both of which are incorporated by reference herein in their entireties). Since *jointless* tomato fruits are "stemless" when harvested, they are highly desirable for products such as tomato juice and canned tomatoes.

[0130] Advances made in understanding floral abscission in *Arabidopsis* are applicable to crops in which it would be desirable to control abscission. Although differences exist between plant species as to which plant organs undergo abscission and the timing of abscission, many aspects of abscission zone differentiation and the abscission process itself are conserved between *Arabidopsis* and important crop species, as has already been demonstrated in numerous studies of the abscission process. Cell wall hydrolyzing enyzmes implicated in abscission such as cellulases and polygalacturonases have been identified in many plant species studied so far (del Campillo and Bennett, 1996, *Plant Physiol* 111: 813-820; Koehler *et al.* 1996, *Plant Mol Biol* 31: 595-606; Trainotti *et al.*, 1997, *Plant Mol Biol* 34: 791-802; del Campillo, 1999, *Curr Top Bev Biol* 46: 39-61; Torki *et al.*, 1999, *Mol Gen Genet* 261: 948-952, all of which are incorporated by reference herein in their entireties).

[0131] Furthermore, vital components of the ethylene response pathway, such as ethylene receptors, also show conservation in distantly-related plant species (Mita *et al.*, 1998, *Plant Cell Physiol* 39: 1209-1217; Sato-Nara *et al.*, 1999, *Plant Physiol* 120: 321-330, both of which are incorporated by reference herein in their entireties). For example, the *never-ripe* mutant of tomato corresponds to an ethylene receptor, and exhibits delayed floral abscission as well as ripening defects (Lanahan *et al.*, 1994, *Plant Cell* 6: 521-530; Wilkinson *et al.*, 1995, *Science* 270: 1807-1809, both of which are incorporated by reference herein in their entireties). Dominant mutant forms of an *Arabidopsis* ethylene receptor, *ETR1*, when introduced into other species, such as tomato and petunia, also cause delayed floral abscission just as in *Arabidopsis* (Wilkinson *et al.*, 1997, *Nature Biotechnology* 15: 444-447, which is incorporated by reference herein in its entirety).

[0132] Tremendous potential exists for abscission-related gene discovery in *Arabidopsis*. The molecular genetic approaches to explore abscission zone development and the genes involved will be greatly facilitated by the advantages of studying this model plant system and then applying the knowledge gained from *Arabidopsis* to other plants.

Example 6

NEVERSHED effects on resource allocation to fruits

[0133] Preliminary analyses suggested that the fruits of *nevershed* mutants are somewhat shorter than wild-type fruits. In order to determine whether one of the purposes of floral organ abscission is redistribution, or allocation, of energy resources to the developing fruit, careful measurements are made of *nevershed* fruits from mutants backcrossed at least three times to wildtype. Measurements made include fruit length, diameter, ratio of length to diameter, cell wall thickness, seed number, seed weight, nitrogen content, carbon content, water content, and any other parameters that suggest how resources are allocated in *nevershed* and wildtype fruits. A difference in various parameters between wildtype fruits and *nevershed* fruits from near-isogenic backgrounds would indicate that floral organ abscission has a role in determining the allocation of energy resouces to developing fruits.

Example 7

Abscission zone development

[0134] The development of the abscission zone of *nevershed* and wild-type flowers is examined by tissue sectioning and by scanning electron microscopy, as described in Liljegren *et al.* (2000, *Nature* 404, 766-770, which is incorporated by reference herein in its entirety), and is tested for chemical composition, and physical and mechanical properties. For histological staining, tissue from wild-type and *nevershed* mutants is fixed, sectioned and stained with toluidine blue, as described by Mixukami & Ma (1992, *Cell* 71: 119-131, which is incorporated by reference herein in its entirety) with minor modifications. For lignin analysis, sections are stained for 2 minutes in a 2% phloroglucinol solution in 95% ethanol, then photographed in 50% hydrochloric acid. For scanning electron microscopy, tissue from wild-type and *nevershed* mutants is fixed for approximately 4 h at 25 °C in FAA (50% ethanol, 5% glacial acetic acid, 3.7% formaldehyde) and prepared for scanning electron microscopy. Samples are examined in a Cambridge S360 scanning electron microscope using an accelerating voltage of 10 kV. Breakstrength testing of the floral organs of the *nevershed* mutant and the floral organs of the wild-type is performed to test physical and mechanical properties of each type.

[0135] The abscission zones of wild-type *Arabidopsis* floral organs, like those of other plants, consist of small, densely cytoplasmic cells, and appear to be only a few cell layers thick. Abscission zone development of the *nevershed* mutant is blocked at an early stage. Expression analyses of abscission zone molecular markers in the *nevershed* mutant as described below is used to assist in pinpointing the stage at which abscission zone development is first disrupted.

Example 8

Temporal and spatial expression profiling

[0136] Temporal and spatial expression profiling of the NEVERSHED gene is carried out in plants transformed with an expression vector having the NEVERSHED promoter region and the β-glucuronidase (GUS) reporter gene, and tissues were fixed, sectioned, and stained as described in Blazquez *et al.*, (1997, *Development* 124: 3835-3844, which is incorporated by reference herein in its entirety), with minor modifications, and in Ferrandiz *et al* (2000, *Science* 289: 436-438, which is incorporated by reference herein in its entirety).

Example 9

Expression profiling

[0137] Candidates for genes that act downstream of NEVERSHED,or are otherwise in a regulatory relation with NEVERSHED, are identified using high-density Affymetrix chips representing the *Arabidopsis* genome. The expression profiles wild-type, *nev-1* and *nev-2 Arabidopsis* in the same genetic background are compared, and differences in expression between wild-type and *nevershed* mutants are used to determine genes whose expression is affected by the expression of the *NEVERSHED* gene.

[0138] The first experiment uses first-generation chips containing almost two-thirds of the *Arabidopsis* genome, which is about 13-16,000 out of 20-25,000 predicted total genes. Next, chips having greater coverage of the *Arabidopsis* genome are used. Results from chips that have incomplete coverage of the genome, but in combination provide complete coverage of the genome, are combined to determine the global expression profile of wildtype and *nevershed* mutants. In another experiment, chips containing the entire

Arabidopsis genome are utilized to determine the effects of nevershed mutants on the expression of all genes in the *Arabidopsis* genome.

[0139] In order to make probes for chip hybridization, poly A^+-RNAs are isolated from the floral abscission zone regions of *nevershed* mutants and wildtype plants using standard procedures (Carninci *et al.*, 1996, *Genomics* 37: 327-336; Theologis *et al.*, 1985, *J Mol Biol* 183: 53-68, both of which are incorporated by reference herein in their entireties), from plants at a number of critical developmental stages determined through mutant characterization. RNA probe are biotinylated, and the labelled probes are incubated with the chip under conditions favorable for hybridization. The array is stained with a phycoerythrin-streptavadin conjugate and then scanned with a laser confocal scanning device that detects and records the amount of fluorescence, as described by Wodicka *et al.* (1997, *supra*, which is incorporated by reference herein in its entirety).

Example 10

Analysis of abscission zone molecular markers in the *nevershed* mutant

[0140] Molecular marker for the abscission zone are selected from the over 1800 *Arabidopsis* enhancer trap lines that show staining patterns in the abscission zone (Campisi *et al.*, 1999, *Plant J* 17: 699-707, which is incorporated by reference herein in its entirety). Markers for numerous stages of abscission zone differentiation are represented within this collection. Molecular markers from gene- and enhancer-trap collections are crossed to *nevershed* mutants, to provide evidence of altered molecular differentiation can be detected even before phenotypic defects become visible. Tissues are stained as described by Campisi *et al.* (1999, *supra*, which is incorporated by reference herein in its entirety) and the lines which show activity at crucial developmental stages and/or crucial locations are selected.

[0141] Markers which are expressed specifically at the bases of developing floral organs are detected by staining as early as stage 6 and markers expressed as late as stage 17 are detected by staining in abscission zone scar tissue. Abscission zone markers representing different temporal profiles are crossed to *nevershed* mutants and are used to pinpoint the stage at which abscission zone development is first affected in these mutants.

Example 11

Molecular characterization of abscission zone molecular markers

[0142] Molecular characterization of abscission zone markers affected by the *nevershed* mutation is carried out to uncover additional genes involved in abscission zone development. As described previously, a set of ten markers representing different temporal abscission zone profiles are crossed to the *nevershed* mutant. In a separate experiment, these markers are also be crossed to an ethylene-insensitive mutant, *ein2*, in which floral abscission is delayed. Because *EIN2* is an integral member of the ethylene-response pathway abscission zone markers which are regulated by this pathway will show delayed expression profiles in *ein2* mutant flowers compared to wildtype. This experiment demonstrates that most if not all markers which are regulated by the ethylene-response pathway also show altered expression profiles in the *nevershed* mutant.

Example 12

Screening T-DNA populations for additional abscission mutants

[0143] Loss-of-function mutants are identified by screening DNA insertional lines. Genes that act downstream of *NEVERSHED,* identified through microarray analyses and characterization of abscission zone markers as described above, are used in screens of a collection of T-DNA insertional lines containing 150,000 individual T-DNA tagged lines, and the estimated equivalent of 225,000 insertions. Gene-specific oligonucleotides and oligonucleotides from the T-DNA borders, DNA pools of this collection are screened as described by Krysan *et al.*, (1996, *Proc Nat Acad Sci USA* 93: 8145-8150, which is incorporated by reference herein in its entirety). Pools that show hybridizing bands are broken down until individual lines are positively identified. Seeds from identified T-DNA tagged mutants are planted out, and the phenotypes of the resulting plants analyzed.

Example 13

ARF GAP activity of NEVERSHED

[0144] NEVERSHED is expressed in a plant cell expression system and purified using the method of Makler *et al* (1995, *Jnl Biol Chem* 270: 5232-5237, which is incorporated by reference herein in its entirety) modified for extraction from plant cells. The

ARF-directed GAP activity of NEVERSHED is measured by at the method of Makler *et al* (1995, *Jnl Biol Chem* 270: 5232-5237, which is incorporated by reference herein in its entirety), where the assay measures a single round of a GTPase reaction. ARF1 is loaded with ^{32}[α-P]GTP in the presence of dimyristoyl phosphatidylcholine (DMPC) and cholate and using a relatively high ARF concentration in order to achieve high loading efficiency. The loading reaction mixture contains ARF1 (0.5 mg/ml),^{32}[α-P]GTP (0.2 mCi/ml, 0.25 μM), 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP plus ATP/GTP regeneration system (5 mM phosphocreatine and 50 μg/ml creatine phosphokinase), 25 mM MOPS buffer, pH 7.5, 150 mM KCl, and a mixture of DMPC and sodium cholate, added last from a 10X stock to give 3 mM and 1 mg/ml, respectively. Loading is carried out for 90 min at 30 °C, and the preparation was divided into small aliquots and stored at -80 °C. Filter binding assays show that between 30 and 60% of the ^{32}[α-P]GTP becomes associated with different preparations of recombinant ARF.GAP activity was assayed in a final volume of 10 μl in the presence of 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP plus the above mentioned ATP/GTP regeneration system, 25 mM MOPS buffer, pH 7.5, 0.5 mM unlabeled GTP, 0.1 unit/ml guanylate kinase, and 1 μl of ^{32}[α-P]GTP-loaded ARF. Following incubation for 15 min at 30 °C, reactions are boiled for 1 min to release the nucleotides from ARF, and the nucleotides are separated by thin layer chromatography on PEI-cellulose sheets, developed with 1.2 M Tris-Cl, pH 7.4. ^{32}[α-P]GDP formation is determined by autoradiography or by cutting the GDP and GTP areas, and determination of radioactivity is made using Cerenkov radiation.

[0145] Data are presented as the percentage of ARF-bound ^{32}[α-P]GTP that is converted to ^{32}[α-P]GDP. Background values are 4-5%, and these values do not increase during incubations in the absence of a GAP. Where appropriate, GAP activity is assessed by carrying out serial dilutions of the sample, and specific activity is calculated at the protein concentration that results in 50% hydrolysis of ARF-bound GTP.

Sequences of NEVERSHED and *Arabidopsis* homologs

[0146] There are at least 5 other *Arabidopsis* genes containing homology to the NEVERSHED ARFGAP domain. For example, MKP6.22, is shown in SEQ ID NO: 9 (genomic sequence) and SEQ ID NO: 10 (amino acid sequence). The amino acid sequence of MKP6.22 is 76% identical to NEVERSHED at the amino acid sequence level in the ARF GAP domain. Other genes having homology to the NEVERSHED ARF GAP domain include the F13M22.5 homolog (SEQ ID NO: 11 (genomic sequence) and SEQ ID NO: 12 (amino acid sequence)), the F17A17.28 homolog (SEQ ID NO: 13 (genomic sequence) and SEQ ID NO: 14 (amino acid sequence)), the F5K20.10 homolog (SEQ ID NO: 15 (genomic sequence) and SEQ ID NO: 16 (amino acid sequence)), and the MZA15.17 homolog (SEQ ID NO: 17 (genomic sequence) and SEQ ID NO: 18 (amino acid sequence)).

[0147] The genomic sequence of wild-type NEVERSHED (MDK4.13), as displayed in the complementary direction, is shown in SEQ ID NO: 8. The exon locations (complementary direction) are as follows: 0024...0192, 0306...0382, 0492...0731, 0957...1129, 1264...1477, 1744...1975, 2093...2183, 2270...2324, 2724...2814, 2902...2960, 3778...3828. The predicted promoter region (complementary direction): occurs at approximately 3829-5567 of SEQ ID NO: 8.

[0148] The genomic sequence of MKP6.22 is shown in SEQ ID NO: 9. The exon locations are as follows: 678...728, 820...878, 969...1059, 1142...1196, 1285...1375, 1471...1672, 1822...1911, and 2052...2156. The promoter region is located at nucleic acid numbers 49 through 677. The polypeptide sequence of MKP6.22 is shown in SEQ ID NO. 10.

[0149] The genomic sequence of the F13M22.5 homolog, displayed in the forward direction, is shown in SEQ ID NO: 11. The genomic sequence, displayed in the complementary direction, is shown in SEQ ID NO: 19. The exons (complementary direction) are located at nucleic acid numbers 41...547, 982...1059, and 1168...1953 of SEQ ID NO: 19. The promoter (complementary direction) is located at approximately 1954-3138 of SEQ ID NO: 19. The polypeptide sequence of F13M22.5 is shown in SEQ ID NO: 12.

[0150] The genomic sequence of the F17A17.28 homolog is shown in SEQ ID NO: 13. The exons are located at position numbers 1026...1032, 1264...1353, 1447...1531, 1609...1684, 1778...1941, 2022...2226, 2310...24266, 2506...2601, and 2677...2958. The

promoter region is located at approximately position 7 to 1025. The polypeptide sequence of the F17A17.28 homolog is shown in SEQ ID NO: 14.

[0151] The genomic sequence of the F5K20.10 homolog, displayed in the forward direction, is shown in SEQ ID NO: 15. The genomic sequence, displayed in the complementary direction, is shown in SEQ ID NO: 20. The exons (complementary direction) are located at positions 266...355, and -481...40 of SEQ ID NO: 20. The promoter region (complementary direction) is located at position 356 through approximately position 5636 of SEQ ID NO: 20. The polypeptide sequence of the F5K20.10 homolog is shown in SEQ ID NO: 16.

[0152] The genomic sequence of the MZA15.17 homolog, displayed in the forward direction, is shown in SEQ ID NO: 17. The genomic sequence, displayed in the complementary direction, is shown in SEQ ID NO: 21. The exons (complementary direction) are located at positions 25...132, 224...325, 411...536, 629...889, 975...1398, 1605...1723, and 1824...1892 of SEQ ID NO: 21. The promoter region (complementary direction) is located at position 1893 though approximately 4305 of SEQ ID NO: 21. The polypeptide sequence of the MZA15.17 homolog is shown in SEQ ID NO: 18.